Universidade de Aveiro Departamento de Biologia
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Is Genistein a Threat to Male Reproductive Potential?

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Será a Genisteina uma Ameaça para o Potencial Reprodutivo Masculino?

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Marco G. Alves, Investigador Assistente/Investigador Principal na Unidade Multidisciplinar de Investigação Biomédica, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, do Professor Doutor Mário Sousa, Professor Catedrático, Director do Laboratório de Biologia Celular no Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto e da Professora Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro.

“All limits are self imposed.” - Icarus

O júri

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Palavras-chave

Estrogénio, Genisteina, Infertilidade, Potencial Reprodutivo Masculino, Fitoestrogénios, Isoflavonas de Soja, espermatogénese.

Resumo

A genisteína é um dos fitoestrogénios mais abundantes na soja. Devido à sua similaridade química com os estrogénios endógenos, a genisteína pode se ligar aos receptores de estrogénio (ERs) presente nas células e desencadear uma resposta estrogénica. A sua ação estrogénica pode potencialmente interferir com o funcionamento normal de vários sistemas no organismo humano, incluindo o sistema reprodutivo que é altamente regulado pelas hormonas sexuais. Tem-se verificado um crescente interesse em usar genisteína para tratamento e prevenção de doenças como o cancro, osteoporose, doenças cardiovasculares, alívio dos sintomas de menopausa, entre outros. Há ainda um o aumento do consumo de soja em populações ocidentais, como substituto da proteína animal e é usado para produzir alimentos para bebés sendo fatores preocupantes porque os efeitos dos fitoestrogénios no organismo humano ainda estão sob discussão. Por outro lado, estudos animais são contraditórios e estudos humanos são escassos.

No presente estudo avaliou-se a ação da genisteína em células de Sertoli humanas (hSCs). Essas células são fundamentais para uma espermatogênese correta, uma vez que proporcionam suporte físico e nutricional às células germinativas. Biópsias de seis pacientes com espermatogênese conservada, foram recolhidas numa clínica de tratamento de fertilidade e culturas primárias de células de Sertoli humanas foram semeadas e mantidas até atingirem confluência. Atingida essa condição, as células foram tratadas com meios contendo diferentes concentrações de genisteína, durante 24h. Foram realizados diversos testes de citotoxicidade. Não foram detectadas alterações nas hSCs tratadas com genisteína. Conclui-se então que este composto não é tóxico para estas células. Posteriormente, efetuou-se a análise de uma das vias metabólicas mais relevantes nestas células, a glicólise, que origina piruvato que posteriormente é utilizado para a produção de lactato. Esse substrato é uma importante fonte energética para células germinativas. No entanto, a absorção e excreção de diferentes metabolitos desta via, além da atividade enzimática da lactato desidrogenase, não mostraram variações significativas após a exposição das hSCs a genisteína.

Também se avaliou a influência da genisteína nas taxas de stress oxidativo em hSCs, uma vez que a produção de espécies reactivas de oxigénio é comum durante o metabolismo celular e, quando não são eficientemente eliminadas, podem causar danos irreversíveis em diferentes estruturas celulares podendo até levar à morte celular. Os resultados do presente estudo demonstram que a genisteína não influenciou as taxas de stress oxidativo. Em suma, conclui-se que não foram identificados efeitos nocivos por parte da genisteína sobre os parametros analisados nas hSCs. No entanto, isso não significa que a genisteína, e outros fitoestrogénios, sejam seguros para o resto do sistema reprodutivo. Por esta razão deverão ser efetuados vários estudos no futuro para o esclarecimento mais amplo da sua ação.

Keywords Estrogen, Genistein, Infertility, Male Reproductive Function, Phytoestrogens, Soy Isoflavone, Spermatogenesis.

Abstract

Genistein is one of the most abundant phytoestrogens in soybeans. Because of its chemical similarity to endogenous estrogens, genistein can bind to estrogen receptors (ERs) present in cells and mediate an estrogenic response. Its estrogenic action may potentially interfere with normal functioning of various systems in the human organism, including the reproductive system which is highly regulated by sex hormones. The increasing interest in using genistein for treatment and prevention of diseases such as cancer, osteoporosis, cardiovascular diseases, relief of menopause symptoms, among others. As well as the increasing consumption of soy in Western populations as a substitute for animal protein and its use to produce food for infants are worrisome factors because the effects of phytoestrogens on the human organism are still a matter of intense debate. Animal studies are contradictory and human studies are scarce.

The aim of the present study was to evaluate genistein safety on human Sertoli cells (hSCs) in vitro. These cells are pivotal for successful espermatogenesis since they provide nutritional and physical support to sperm germ cells. Biopsies from six patients, with conserved espermatogenesis, were chosen at an infertility clinic and primary cultures of human Sertoli cells were cultured and maintained until reaching confluence. Then they were treated with different genistein concentrations during 24h. Different citotoxicity test were perfomed. No disturbances were found in hSCs treated with genistein. Therefore, we conclude that this compound is not cytotoxic to these cells. Subsequently, the analysis of one of the most relevant metabolic pathways, glycolysis, in these cells was made, which originates pyruvate, that in turn, later, is used for the production of lactate. This substrate is an important energy source for germ cells. However, the absorption and excretion of different metabolites of this pathway, in addition to the enzymatic activity of lactate dehydrogenase, did not show significant variations after exposure of hSCs to genistein. Also genistein influence on oxidative stress rates in hSCs was evaluated, since reactive oxigene species (ROS) production is common during cell metabolism and if not eliminated efficiently it can cause irreversible damage on different cell structures and may even lead to cell death. The results of this study demonstrate that genistein did not influence oxidative stress rates.

Alltoghether, we can conclude that no harmfull effects, on the parameters analyzed, were caused by genistein on hSCs. Altough it does not mean that genistein, and other phytoestrogens, are safe for the remaining of the reproductive system more studies regarding this subject are needed for a better understanding of their mechanisms of action.

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List of Abbreviations

17β-HSD – 17β-Hydroxysteroid Dehydrogenase

4-HNE – 4-hydroxynonenal

ATP – Adenosine Triphosphate

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNP – 2,4-dinitrophenyl

DNPH – 2,4-dinitrophenylhydrazine

ECF – Enhanced Chemi Fluorescent substract

ECL – Enhanced Chemi Luminescent substract

ER – Estrogen Receptor

ERα – Estrogen Receptor α

ERβ – Estrogen Receptor β

FBS – Fetal Bovine Serum

FSH – Follicle-Stimulating Hormone

GPER – G-Protein-Coupled Estrogen Receptor 1

HEPES – Hydroxyethyl Piperazineethanesulfonic Acid

^{1}H-NMR – Hydrogen-1 Nuclear Magnetic Resonance

hSCs – Human Sertoli Cells

ITS – Insulin-Transferrin-Sodium

JC-1 – 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimi-dazolylcarbocyanine iodide

LDH – Lactate dehydrogenase enzyme

LH – Luteinizing Hormone

MTT – Tetrazolium Dye MTT Assay

M-PER – Mammalian Protein Extraction Reagent

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NTP – National Toxicology Program

OS – Oxidative Stress

PBS – Phosphate-buffered Saline Solution

PVDF membrane – Polyvinylidene Difluoride Membrane

SDS - Sodium Dodecyl Sulfate

SHBG – Sex Hormone Binding Globulins

SIF – Soy Infant Formula

SRB – Sulforhodamine B assay

TCA cycle – Tricarboxylic Acid cycle

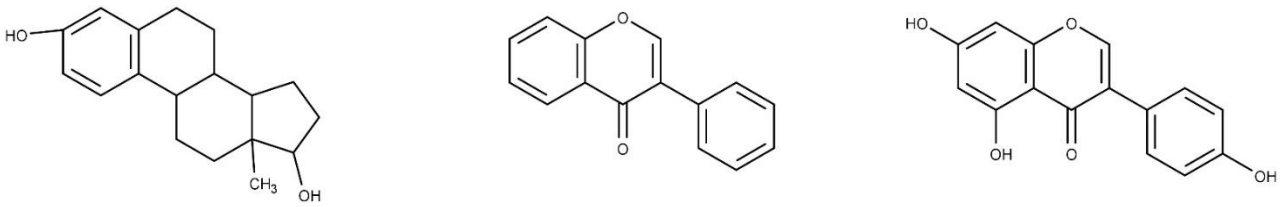
I. Introduction

1. Introduction

In the last decades, there has been an increased interest in phytoestrogens, because of growing body of evidence suggesting that these compounds can be potentially beneficial to human health and may even prevent or treat diseases such as cancer, cardiovascular diseases, osteoporosis, slow down skin aging, boost memory and alleviate menopausal symptoms (1-3). However, its similarity to endogenous estrogens arises concerns about possible disruptive effects that it can cause, especially in the organs and systems regulated by sexual hormones.

Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen, a class of compounds that include several groups of non-steroidal estrogens easily found in the plant kingdom. These compounds, due to their chemical similarity with endogenous estrogens, such as 17β-estradiol as shown in Fig.1, can bound to mammalian estrogen receptors and trigger an estrogenic response, although a weak one (1, 4, 5).

Animal studies have already shown disruptive effects of phytoestrogens on fertility. One of the first reported cases (1946) was in sheep, where it was found that sheep grazing in fields rich in clover (*Trifolium pratense*) developed infertility. Apparently, clovers are rich in a phytoestrogen subgroup called isoflavones, of which the most abundant, in this case, was formononetin. The diet rich on those compounds led to dysfunction in the reproductive system of sheep (6). Humans generally do not consume clover but consume many products derived from soy, which is also rich in isoflavones, particularly daidzein and genistein (7). The increasing use of soy in food industry as an alternative source of protein (5), as well as its use as substitute for animal products in the diet of many people arises several relevant concerns due to the lack of conclusive information on the effects and risks, for the use (or misuse), of phytoestrogens on human health.



1 Estradiol 2 Isoflavone 3 Genistein

Figure 1 - Chemical structure of 17β-estradiol in relation to isoflavones general chemical structure and to genistein. Genistein mainly consists of two aromatic rings linked to a heterocyclic pyrane ring in the center, and three hydroxyl groups which are required for binding to the ER. Distance between these groups is similar to those of 17β-estradiol.

2. Estrogens and Male Reproduction

For long time estrogens were regarded as female sex hormones. However, it was shown that androgens are metabolized to estrogens in both sexes and that the latter play an important role by themselves but also as mediators of androgens-associated action in many physiological functions. In addition, estrogens are responsible for important processes including growth, cardiovascular function, bone mineralization, brain masculinization and sexual behavior, by hormone action on different target tissues (8-12). In males, most estrogens are produced from androgens in a conversion mediated by aromatase, which is mainly present in Leydig cells. However, aromatase activity was also detected in germ cells, mature spermatozoa and Sertoli cells in the reproductive tract and also in brain, adipose tissue and skin (13, 14). Estrogens circulate in the plasma bound to SHBGs and enter in the cells via passive diffusion (15). They act by binding to their specific receptors, ER, which exists in two functional isoforms, namely ERα and ERβ, localized at cytosolic and nuclear level. When activated, these receptors interact with a specific DNA sequence, through DNA binding domain, and activate or repress the expression of that gene (16). Both receptors are expressed in male reproductive organs, although differently distributed in different cell types. There are differences in the location of ERs in the male reproductive tract between different species, even among the nearest ones (17-19). However, Sertoli cells and Leydig cells appear to preserve their similarities, among species, in the type of ERs they possess. For example, ERα is always present in Leydig cells and ERβ in both, plus in spermatozoa ERβ is present too (17-21).

In addition to the two classical estrogen receptors, there is yet another receptor that can trigger a signal in the presence of estrogens, G-protein-coupled ER1, GPER, also known as GPR30, a receptor belonging to a G-protein coupled receptors

superfamily. Its discovery is quite recent and its functionality is still a matter of debate (22). It is known that this receptor is present in different tissues including the male reproductive organs. It can be found in Sertoli cells and germ cells and is especially overexpressed in seminomas, the most frequent testicular germ cell cancer (23). It has been shown that in the presence of estrogen this receptor can trigger estrogenic responses in cells that have ERα and ERβ absent (22, 24, 25). Knockout animals for this receptor do not exhibit any abnormalities in the reproductive tract and are fertile, however some of these animals suffer from impaired production of T cells in the thymus, have impaired glucose tolerance, elevated blood pressure and reduced bone growth (26-30).

Studies focused on ERα knockout mice showed that absence of this receptor leads to infertility and progressive degeneration of testes and efferent ductal epithelium. Those mice had normal fetal and afterbirth development until puberty, time when was observed an increase in testis volume and weight followed by complete atrophy at around 150 days age (15, 31). Another effect caused by the absence of ERα was the loss of reabsorption function in epithelial cells of efferent ductules, thus causing an accumulation of fluid into the lumen and exerting back pressure into the testis, probably the cause for initial testis weight gain. The accumulation of this fluid caused a disruption on spermatozoa development and semen concentration, negatively affecting the fertility of the mice (32). Spermatozoa with abnormal morphology were also observed in ERα knockout mice and these spermatozoa had flagella separated from their heads. With age, spermatozoa in these mice progressively decreased in number, motility and fertilizing ability, both *in vitro* and *in vivo* (15, 31). Sexual behavior was also affected with males showing less interest in females. Thus, number and frequency of mounts were significantly lower when compared to the wild type mice (31, 33).

Interestingly, ERβ knockout mice did not presented significant alterations in development, while fertility and mating rates were also maintained normal. Only in aging mice was noted hyperplastic prostate and bladder (34). Later, scientists were able to create mice lacking both ERs and the characteristics presented were very similar to that of ERα knockout mice which highlights again the major relevance of ERα for male fertility (35).

Other studies were performed in mice lacking aromatase to unravel a possible role for this enzyme in male reproduction. Initially those mice had normal fertility, sperm parameters and reproductive tract morphology. However, throughout lifetime, occurred a decrease in spermatozoa count and sperm motility and loss of sperm fertilizing potential. Spermatozoa count decrease was mainly due to low survival rates of round

spermatids, which leads to a progressive impairment of spermatogenesis. Number of mounts was also decreased contributing to the lower fertility rates observed in these mice (36-38).

In human patients, congenital estrogen deficiency and estrogen resistance is suggested to be not so detrimental to reproductive function as in mice. However, these individuals share a common trait with estrogen resistant mice, impaired sperm motility, and both aromatase-deficient mice and men have premature germ cells arrest, which is responsible for reduced sperm count (39). These individuals undergo normal puberty, have normal external genitalia and prostate and no gender-identity disorders. However, they exhibited impaired bone mineralization leading to osteoporosis/osteopenia and abnormal epiphyseal maturation associated with continuing linear growth and bone pain, eunuchoid body proportions, insulin resistance and impaired lipid and glucose metabolism. Gonadotropins and testosterone levels range from normal to elevated, while estrogen is below the range of detection (9, 40-46). It is important to note that phenotypes displayed on knockout mice and in human patients, sharing the same deficiencies either in ERα or aromatase, were not exactly the same, especially in behavioral aspects (39). These results show that data obtained with animal studies cannot be always directly extrapolated to humans, particularly concerning hormonal response and signaling. Nevertheless, all studies clearly support that estrogens are key players for male reproduction.

3. Phytoestrogens Source

In nature, phytoestrogens occur in different vegetables, fruits and whole grains commonly consumed by humans. Isoflavones are mostly abundant in *Fabaceae* family, mainly soybeans (*Glycine max*), they can also be found in other legumes such as the common bean (*Phaseolus vulgaris*). Other plants rich in isoflavones are red clover (*Trifolium pratense*), chickpeas (*Cicer arietinum*), alfalfa (*Medicago sativa*), kudzu (*Pueraria lobata*), licorice (*Glycyrrhiza glabra*) and some Chinese medicinal herbs, such as *Genista tinctoria* and *Sophora subprostrala* (1, 47-52).

Soy was not originally cultivated for human consumption, but for its nitrogen fixing abilities because rice, which is the base of Asian diet, is nitrogen-depleting to soil. Also, soy is difficult to digest, and it was incorporated into the Asian diet mostly in fermented forms, such as soy sauce, miso, tempeh and natto. Tofu is also highly

consumed in the East but in contrast to the other soy products it has more phytoestrogens since it does not undergo fermentation, because fermentation is a process which makes soy products to lose some of their phytoestrogens. However, soy products highly consumed in the West, like soy milk, soy yogurts, soy-based protein powders, beverages, baked goods, tofu, and others do not undergo fermentation and thus, they retain most of their phytoestrogens (5, 53).

Soy infant formula (SIF) is another soy based product that raises concerns. SIF is an infant food made using, among different components, soy protein and it has become quite popular as a replacement for human milk or cow milk formula, especially for lactose-intolerant babies or in vegan families. Based on 2009 market data, sales of soy formula range from 2-7% of total infant formula sales in Europe and about 12% in the United States (National Institute of Environmental Health Sciences – U.S.). Although there have been no specific health problems documented in human infants receiving soy formula, its safety has been questioned duet to its high isoflavone content (54). It was shown that infants fed on SIF have phytoestrogen intake 6-11 fold higher per kg of body weight than that seen in adults consuming a diet rich in phytoestrogen (55). Such high levels of phytoestrogens are expectable because SIF represents 100% of an infant diet while adults consume a variety of different products, so even if a diet in adults is high in soy, its total percentage on diet will not be as high as on infant fed on SIF. Plasma levels of isoflavones on SIF fed infants were 13 000-22 000 times higher than those of endogenous estradiol, which is worrisome because prolonged neonatal exposure to such high levels of phytoestrogens may potentially disrupt normal development and the function of reproductive organs, since it is in this age that testes are structurally organized and Sertoli cell and spermatogonia numbers are established, which will ensure normal spermatogenesis initiated at puberty (55, 56).

Thus, The National Toxicology Program (NTP) Center for Evaluation of Risks to Human Reproduction, convened an expert panel on 2009 to evaluate the safety of SIF, based on opinion of experts and a discussion on the scientific publications available at the moment of the meeting. The NTP used a five-level scale in its conclusions to characterize the likelihood of an adverse human health effect resulting from exposure to a substance or chemical, in this case soy infant formula. The levels were divided in concern: serious, regular, some, minimal or negligible. Under this evaluation, the NTP expressed minimal concern for soy infant formula consumption (National Institute of Environmental Health Sciences – U.S.). Nevertheless, this is still a matter of debate and

phytoestrogens sources and mechanisms of action are on spotlight and remain a focus of research.

4. Phytoestrogens Metabolism

Phytoestrogens are known to be metabolized by intestinal bacteria and then absorbed. They are also conjugated in the liver, circulate in plasma and excreted in the urine (3). Thus, the overall effects of phytoestrogens can be influenced by any of these metabolic pathways.

Isoflavones mainly exist in soy foods as glycoside conjugates, genistin for genistein and daidzin for daidzein (57-59). They are highly polar and water-soluble which difficult their absorption by intestinal epithelium. However, in the intestine by action of both intestinal mucosal and bacterial β-glucosidases and lactase-phlorizin hydrolase, isoflavone glycosides are hydrolyzed to respective aglycones, that are directly absorbed and undergo hepatic metabolism, or further metabolized by intestinal microflora into other metabolites (2, 59). In the colon, daidzein can be further metabolized to equol (about 70%) or *O*-desmethylangolensin (5-20%), and genistein can be metabolized to *p*-ethylphenol which does not exert estrogenic activity. About 40.1-58.3% of total genistein and genistein administrated are not absorbed and eliminated in the feces. A general metabolic scheme of gut microflora transformation of these compounds is presented in Fig.2 (60-64). When absorbed, isoflavones bind to SHBG and circulate in the organism or undergo enterohepatic circulation. When in liver, they can be conjugated with glucuronide and/or sulfate groups at the 4'- and 7-hydroxyl groups and return to circulation or be excreted in the urine and bile (47, 60, 65-67). Isoflavones in their glycoside form have weaker biological activity than the corresponding aglycones and the capacity of producing these metabolites differs among individuals, mainly due to differences in gut microorganisms (2, 59, 68).

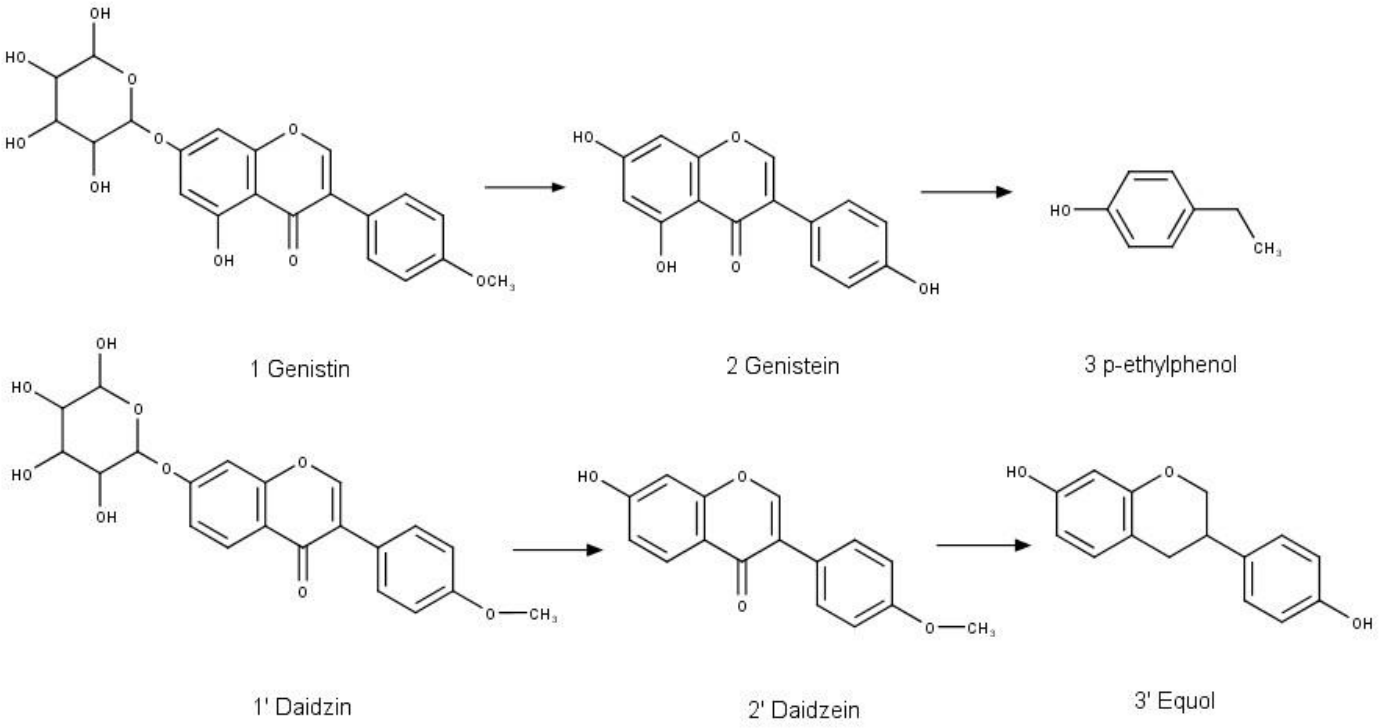


Figure 2 - General chemical transformation of genistein (1,2,3) and daidzein (1',2',3') by the gut microflora. Glycosides are hydrolyzed to the respective aglycones, genistin is converted to genistein and further to 3 p-ethylphenol which does not exert estrogenic activity at this stage. Daidzin is metabolized to daidzein and then to equol which, in contrast to the genistein metabolite exerts estrogenic activity, better than the daidzein itself.

According to recent data, western populations tend to consume more unfermented soy products which in turn are richer in phytoestrogens, but switching to fermented soy products would not be the solution to decrease phytoestrogens intake. Studies have demonstrated that during the consumption of fermented and unfermented soy products, the urinary excretion of isoflavones was approximately the same, suggesting that fermentation increases the availability of isoflavones in soy products (60, 69). However, it is known that diet can also influence phytoestrogen digestion. Diets low in fat and rich in fiber, typical of Eastern populations, are associated with lower estrogen absorption levels in the intestine. This decrease may result due to lower β-glucuronidase activity of intestinal bacteria, observed in individuals consuming this type of diet or due to an increase in SHBG values, trait commonly seen in vegetarian individuals, which probably is caused by the entry of phytoestrogens into the portal circulation in very high amounts and consequent stimulation of this globulin production in the liver, thus clearance rates of estrogens are greater on these individuals (63, 70). On the other hand, Western populations diet is richer in fat and lower in fiber, which makes estrogen absorption easier and even if their daily soy consumption is lower, plasma levels of estrogen/phytoestrogen

absorbed may reach the amount observed in Eastern populations, or even higher. What is worrisome is that soy usage in Western diet is rising significantly, since it is often used as animal protein substitute in ready meals available on supermarkets, either as meat substitute or as bulking agent, also as a promotion of a healthy life style and vegetarianism. This incorporation of soy products in Western diet style, rich in fat and low in fiber, can make these populations be exposed to high levels of phytoestrogens (56, 70). It should also be noted that Eastern population consume high quantity of soy products during centuries and thus, they may have evolved to become more genetically resilient to estrogenic insults whereas Western populations started do consume soy not that long ago, making them more susceptible to possible endocrine disruption effects (56).

5. Chemistry and Action of Phytoestrogens

Main classes of phytoestrogens are flavonoids (flavones, flavonols, flavanones, isoflavonoids), lignans, stilbenoids, chalcones as well as miscellaneous chemicals. However, the greatest estrogenic activity is exerted by isoflavones, flavones, flavonols, flavanones, lignans and chalcones, of which most common are isoflavones and lignans. Special attention is given to isoflavones, where genistein belongs, since they are higly consumed by humans and they include subgroups as isoflavones, isoflavanones, pterocarpanes, coumestans, and others (4, 47, 71, 72).

Chemically, isoflavones are characterized by a 15-carbon (C6-C3-C6) skeleton and have the B-ring linked to the C-3 of the central heterocyclic pyrane ring (C), rather to the C-2 which happens in flavones. Genistein mainly consists of a heterocyclic pyrane ring (C) in the center, which links two aromatic rings (A and B), also has three hydroxyl groups (Fig.3). Hydroxyl groups are pivotal for estrogenicity of a molecule since they form hydrogen bonds with key functional amino acids of the binding part of the receptor. The other requirement to bind ER is the distance between O-O of hydroxyl groups that should be between 10.9Å and 12.5Å, simulating the 3-OH and 17β-OH hydroxyl groups of 17β-estradiol. It is thought that hydroxyl group at C-5 of genistein is responsible for its affinity to ERs, because daidzein, which is identical to genistein only lacks this hydroxyl group, as seen in Fig.3, and essentially has no binding activity to either of ERα or ERβ (4, 47, 73-75).

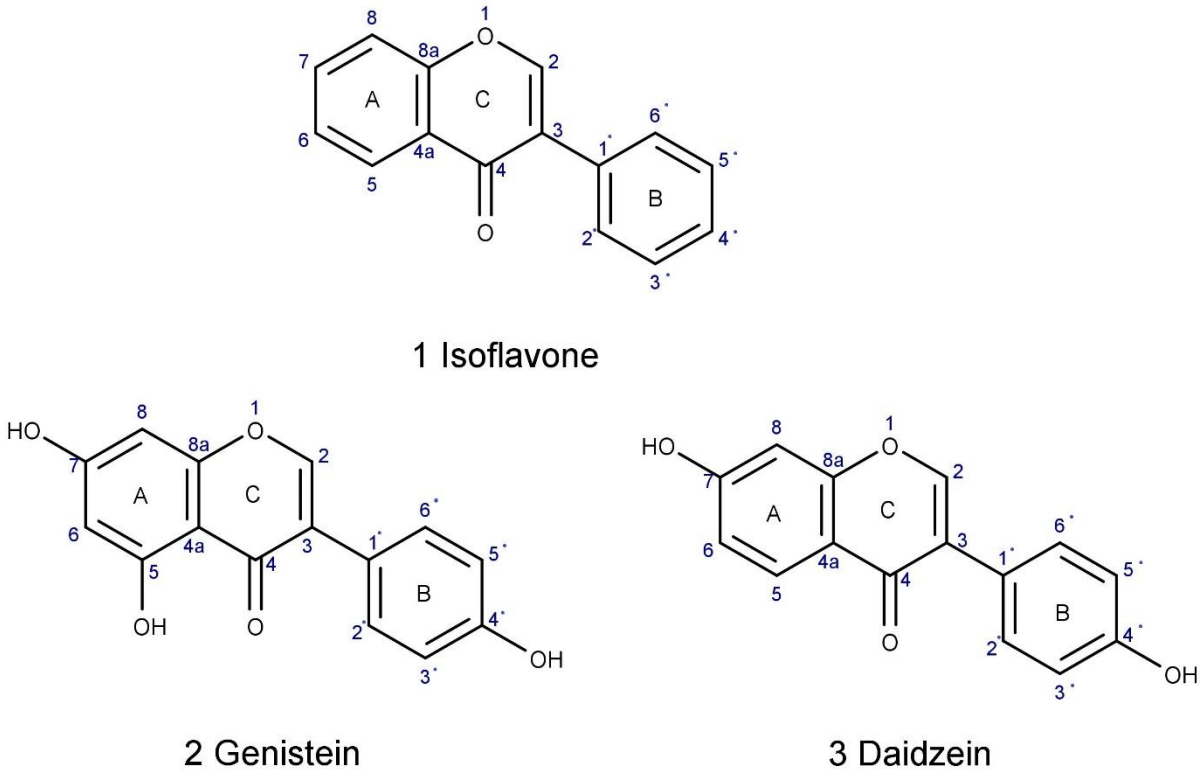


Figure 3 – Isoflavone and daidzein chemical structure in relation to genistein. Both genistein and daidzein belong to the isoflavone subgroup and that is why their chemical structure is the same with only one difference, the hydroxyl group at C-5 which grants genistein its ability to bind to ERs.

As for genistein, it binds to both ER isoforms with moderate affinity but demonstrates a preference for ERβ, with a 30-fold higher affinity (3, 75, 76). Also, genistein is known to exert mixed (agonist/antagonist) properties on both receptors in an organ-dependent manner, depending on the receptor content of specific tissue (ERα and ERβ amount). Antagonist response usually is a result of competition between phytoestrogens and estrogens for ERs (4, 74, 77, 78).

Phytoestrogens not only interact with ERs but can also exert indirect estrogenic response by alternative mechanisms. Phytoestrogens interfere with the synthesis of steroid hormones by inhibiting specific enzymes, such as aromatase, which is responsible for the conversion of androgens to estrogens. Phytoestrogens may also affect the clearance rates of androgens and estrogens by displacing them from SHBG. Apparently, production of these globulins in the liver is stimulated by phytoestrogens and thus, this will affect the total amount of free estrogens in blood circulation and thereby cause an indirect estrogenic effect (4, 70, 71, 79, 80).

6. Phytoestrogens also mediate non-estrogenic effects

Besides their estrogenic activity, phytoestrogens, can also exert non-genomic actions such as tyrosine kinase inhibition (81, 82) which in turn affects cell signaling. Also, genistein was reported to inhibit topoisomerase II activity (83-85) and increase the activity of some antioxidant enzymes, including catalases, superoxide dismutases, glutathione peroxidase and glutathione reductase (86-88). Tyrosine kinase and DNA topoisomerase II inhibition together with antioxidant enzyme stimulation are undoubtedly important factors responsible for phytoestrogen anticarcinogenic activity.

Another phytoestrogen target of these compounds are steroidogenic enzymes. Genistein directly inhibits 3 and 17β-hydroxysteroid (17β-HSD) dehydrogenase activity, thereby disrupting the conversion of androstenedione to testosterone and androstenediol to androsterone (89). Although genistein action on its own on aromatase enzyme is weak (90), its effect on reduction of androgens production by inhibiting 3 and 17β-HSD will indirectly affect estrogen synthesis. Coumestrol and genistein were also shown to reduce 17β-hydroxysteroid oxidoreductase activity which is responsible for conversion of estrone to the more potent 17β-estradiol, thus decreasing the availability of the highly active endogenous estrogens (91).

7. Genistein effect on male fertility: research results in last years from benefits to subfertility or infertility

From the foregoing, we can conclude that estrogens are extremely important for the proper functioning of the reproductive system, not only in female but also in male, and that phytoestrogens can potentially disrupt its normal functioning.

Great interest in phytoestrogens, genistein and daidzein in particular, due to their increasing consumption in Western populations and their potential therapeutic effects made them the focus of many studies in the last decades. Another reason for the increasing study of phytoestrogens is due to their chemical similarity with endogenous estrogens and the controversies that this generated about its safety. There is a need to determine if their estrogenic effects can have disruptive effects on the functioning of our organism or not, and verify if fertility is somehow affected.

Most of studies are performed on rat/mice and only few studies are available about the effects of genistein, and phytoestrogens in general, on human fertility.

7. 1 Rat/mice studies

Rodent studies conditions are very varied, regarding the time of exposure and genistein dose used, as well as the age at the time of administration. Also, results are quite contradictory, even among studies with similar conditions. It should be noted that there are few studies that report beneficial effects of genistein on the reproductive system, most do not find alterations and others affirm that there is a high degree of disturbance in some of the studied parameters. For example, the period of life when animals where submitted to phytoestrogens, genistein inclusively, is crucial and the results attained are different accordingly with the period studied. Prenatal exposure has been shown to negatively affect the fertility of the animal when it reaches adulthood, exposure of adults has also resulted in temporary disturbance of the fertility, however, postnatal exposure, pre-pubertal, during puberty and chronic exposures (over the lifetime of the animal) had both, neutral results (no effects observed) and cases in which some fertility interference occurred, as summarized in Table 1. It is quite interesting that in two multigenerational studies, where several generations of animals were continuously exposed to phytoestrogens (including genistein), their fertility, in general, was unaffected, it was only noted a slight decrease in the number of pups per litter in the second generation, but this effect has been attenuated in subsequent generations (92, 93), thus indicating that probably, continued exposure to phytoestrogens results in some kind of resistance to them.

Weight of the reproductive organs in most cases was unaffected, which seems to ensure the safety of genistein and phytoestrogens in general, however sperm evaluation parameters show some disturbances, as summarized in Table 1. Of the various studies known in the literature, most report a reduced sperm count and motility, and even morphological anomalies were found, this may explain the decrease in offspring. Serum levels of testosterone also appear to be slightly decreased in many cases, on the other hand, FSH and LH do not appear to deviate much from normal levels, cases of slight decrease have been reported as well as cases in which there was no variation (Table 1). Anyway, this did not affect the sexual behavior of these animals, although in one case a decreased interest of males by females was reported, yet this did not affect drastically the fertility in general (94, 95).

Antioxidant power of genistein, and the beneficial effects of this action to the reproductive system has been the only parameter consistently evaluated as positive in most studies (73, 74, 102, 103). Lack of concordant results on the effects of genistein on reproductive system does not support its safety- Jorge M. Naciff et all., 2005, carried out a study in which genistein, among other phytoestrogens, was able to alter the expression of several genes in reproductive organs in a dose dependent manner. About 46 genes were up- or down-regulated by genistein (96), although it is not yet known whether all these modifications have positive or negative effects, is nonetheless a worrying fact that must be taken into account and carefully discussed. Other studies observed a decrease in the production of certain mRNAs in male reproductive organs, such as ERα mRNA in the testes and epididymis (97), although these mice did not have alterations in testis weight, sperm count and sperm motility in adulthood. Decreased levels of mRNA involved in ribosome function in testis, transcripts coding for androgen-response genes in Sertoli cells and Gapd-s (involved in sperm glycolysis and mobility) were significantly reduced, although without significant effects on fertility (95).

Another study in rats reported that genistein aglycones, its most active form, are able to get out of the maternal circulation, pass through placenta and enter fetus blood circulation, and that the difference in concentration of aglycones in fetal serum was only 5-fold lower than maternal. In addition, brain levels of fetal aglycones were very similar to maternal (98), and although studies done with pre-natal and multigenerational genistein exposure did not reveal significant implications for mice fertility, Table 1, the possibility of exposure to phytoestrogens during development still remains a matter of concern and debate.

ABLE 1
Effects of genistein/soy phytoestrogens exposure on rat/mice

		Positive effects	Negative effects	No change
Age and time of exposure	Prenatal		-(96, 99, 100)	
	Postnatal		-(101)	(102)
	Pre and postnatal		-(95, 103)	(104, 105)
	Prepubertal			(106)
	Pubertal		-(107)	(108)
	Adult	+(109)	-(110-113)	
	Chronic		-(114)	(115)
	Multigenerational			(92, 93)
Serum hormone levels	Testosterone	↑*(87) ↑(109)	↓(99, 100, 103, 107, 112, 114)	(95, 102, 116)
	FSH	↑*(87)	↓(103, 112)	(104, 113)
	LH	↑*(87)	↓(103, 104, 112)	(95, 113)
	Estradiol		↓(92)	
Weight	Testis		↓(94, 99, 111)	(97, 104, 105, 108, 115)
	Epididymis		↓(104, 111)	(108, 115)
	Prostate	↑(92)	↓(94, 111)	
	Seminal vesicles	↑(92)	↓(101, 111)	(105)
Sperm parameters	Count	↑(109)	↓(94, 95, 100, 107, 108, 111, 113)	(97, 102, 104, 105, 115)
	Motility	↑(108, 109)	↓(94, 107, 111, 114)	(97, 105)
	Morphology	●(87)	○(111, 112)	(115)
	Viability	↑*(87)	↓(94)	
Other parameters	Antioxidant capacity	◊(87, 88, 117, 118)		
	Gene/mRNA expression alteration	↑”(96)	↓”(95-97, 114)	(105)
	Offspring		↓(95, 100, 114)	(106)
	Sexual behavior		↓[1](94)	(95)
	Anogenital distance		↓(99)	(105, 106)

+ Ameliorating effects on fertility after genistein exposure at different age/developmental stage; - Detrimental effects on fertility after genistein exposure at different age/developmental stage ↑ Increase; ↓ Decrease; ↑* Increase from abnormally low levels to normal levels; ● Increase of the number of morphologically normal spermatozoa; ○ Decrease of the number of morphologically normal spermatozoa; ◊ Reduction of oxidative stress; ↓[1] Reduced interest in mating; ↑” Gene up-regulation; ↓” Gene down-regulation.

7. 2 Human studies

Studies with humans are scarce, which is understandable due to the difficulty of recruiting volunteers and the ethical factors involved. Most of these studies are based on the analysis and comparison of data on the consumption of phytoestrogens and their possible relation with changes in the semen parameters.

A study based on data from an infertility clinic has associated consumption of soy products to a lower sperm count (119). That study is in line with another report conducted in China in which about 1000 men were recruited. That group consisted of men with idiopathic infertility and healthy ones, and high levels of daidzein, genistein and secoisolariciresinol were associated with lower sperm count and motility (120). A meta-analysis conducted by David Lim and Ian C. Shaw (121), on the consumption of phytoestrogens and sperm concentration in the USA and China, also reached a slight association between phytoestrogen consumption and lower sperm count but this association was considered insignificant. It is also not certain whether phytoestrogens may be the cause of this decrease, since there are many other environmental factors that may be involved. Also, administration of 40 mg of isoflavones per day to healthy men for 2 months did not induce any changes in semen parameters, in serum hormone levels or in testis size (122). A study conducted with couples desiring pregnancy, showed that higher levels of genistein and daidzein were associated with increased abnormalities in semen morphology although this did not influence the fertility of the couples (123). Another study with couples from an infertility clinic that were undergoing fertility treatment in which the male partner of the couple was, or not, a consumer of soy products, were evaluated for the success of *in vitro* fertilization, embryo quality, implantation and live at birth, and no relation was found between these parameters and soy consumption (124). Measurement of hormone levels such as testosterone and LH in men who consumed soy or isoflavones whether in the diet or as supplements, also did not found significant variations (78, 125).

It appears that phytoestrogen consumption is associated with a lower sperm count but its association with infertility is difficult to establish, yet there is a case study reporting that phytoestrogens helped to treat infertility, temporarily, in a man with severe oligospermia caused by partial sperm maturation arrest at a spermatidic stage. This man had altered sperm count, motility and morphology and was given 80mg/day of phytoestrogens for 6 months, at 3 months semen parameters significantly improved and resulted in pregnancy, at the end of 6 months semen parameters improved even more. After a period of 6 months wash-out sperm parameters returned to baseline values (126).

In sum, experimental animal models differ from human studies. In humans, the consumption of genistein is not associated with alterations in the male reproductive organs. However, it is verified a trend to a lower sperm count, also a small variation in the hormonal levels and the morphology of the spermatozoa were found, yet, no influence on the fertility of the analyzed individuals. In turn, results of the studies in rats/mice are quite diverse and contradictory, many of the parameters analyzed in the experiments, in half of the cases, present changes and in the other half do not present any alterations. One of the factors in common with human studies is the decrease in sperm count and while in humans there was no influence of the phytoestrogen consumption on fertility, in animals a reduction, on similar studies, was noted. In animals, was also found that the consumption of phytoestrogens can alter the expression levels of several genes in the testis and it can result in up- or in down-regulation depending of the treatment. Another important characteristic found in the reproductive organs of the studied animals was the antioxidant power that phytoestrogens exert, which is indeed a beneficial factor for cellular function.

II. Aims of Project

Since genistein is highly present in human nutrition due to increasing soy consumption and its estrogenic activity is undeniable and safety yet to be proven, we aim to establish a link between genistein exposure and its influence on male reproductive potential. For that we evaluated human Sertoli cell metabolic profile, since these cells are pivotal for spermatogenesis by providing physical and nutritional support for germ cells. Part of the metabolic study was characterized by the lactate production pathway, its major components and the study of the lactate dehydrogenase. This pathway was chosen because lactate is used as main energy source in germ cells.

However, firstly we evaluated the cytotoxicity of genistein in these cells by exposing these cells to different concentrations. The oxidative stress rates in human Sertoli cells exposed to genistein were also evaluated. Overall, we proposed to study the metabolic and oxidative profiles of human Sertoli cells exposed to genistein in order to determine if this phytoestrogen can alter the nutritional support of spermatogenesis, which would have a crucial effect for sperm production.

III. Methods

1. Chemicals

Genistein (G6649-00025MG): Sigma-Aldrich (St. Louis, MO, USA); Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12 (F12) and Fetal Bovine Serum (FBS): Biochrom (Leonorenstr, Berlin, Germany); Insulin-Transferrin-Sodium (ITS): #41400-045 Gibco (Grand Island, NY, USA); JC-1 Molecular Probes: Eugene (OR, USA); LDH Enzymatic Assay Kit and M-PER Mammalian Protein Extraction Reagent: Thermo Scientific (Waltham, MA, USA); Thiazoyl Blue Tetrazolium Bromide (MTT): AMRESCO (OH, USA) were purchased. All other chemicals/reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless specifically stated.

2. Testicular tissue selection and ethical issues

Sertoli cells required for the work were isolated from human testicular biopsies. Testicular biopsies were obtained from patients seeking for fertility treatment in the Center for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal), after informed written consent. Biopsies from patients who had preserved spermatogenesis and were seeking for treatment due to anejaculation (neurologic, psychological or vascular), vas deferens damage or vasectomy were used.

3. Primary culture of human Sertoli cells

Studies were done in accordance with the Helsinki Declaration. The study of patients and biopsies was done according to Guidelines of Local, National and European Ethical Committees in the Center for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal). Human SCs where isolated form cells left in tissue culture plates after patient's treatment by a method previously described by (127). Cells were transferred to culture flasks (Sarstedt, Nümbrecht, Germany) where they grew till reaching confluence. hSCs

culture medium consisted of a mixture of DMEM with Ham F-12 (1:1), suplemented with HEPES, sodium bicarbonate, D-Glucose, 50µg/ml of gentamicin, 50U/mL penicillin, 50mg/mL streptomycin sulphate, 0.5mg/mL fungizone and 10% of inactivated FBS. Medium was changed once in 4 days and cells were kept in an incubator which had the following conditions: 5% CO_2, 95% O_2 at 33-34°C.

4. Preparation of medium containing genistein (treatment medium)

The initial stock of genistein was made with the dissolution of pure genistein in DMSO, organic solvent, to a final concentration of 185 mM. Subsequent dilutions were made from this stock so as to obtain treatment media at a concentration of 10 µM, 1 µM, 0.1 µM, 0.01 µM and 0 µM, the latter did not take genistein but rather the vehicle (i.e. DMSO), as all media were adjusted to have 0.005% of the vehicle (10 µM). The treatment medium consisted of ITS, genistein and 0.005% DMSO. ITS medium is equal to the hSCs culture medium but instead of having FBS it is supplemented with ITS. The concentrations of genistein chosen were based on the literature in which the amount of circulating genistein, in the plasma, was evaluated in different populations with different consumption habits of products containing the same (128). The concentration of 0.01 µM corresponds to a value normally observed in European populations, thus populations that do not commonly consume soy products (129). On the other hand, the concentration 1 µM is observed in Asian populations (130, 131) that usually consume large quantities of soy products, and the concentration of 0.1 µM was chosen as intermediate concentration The concentration of 10 µM is a supraphysiological dose and 0 µM is an absolute control, cells completely devoid from genistein influence (132).

5. Experimental groups

For this work biopsies of 6 different people that had conserved spermatogenesis as mentioned above were chosen, when cells of each biopsy in the culture flask reached confluence they were divided by culture plaques. After cultured, the cells were maintained until reaching a confluence of 90-95%, when this condition was reached the culture medium was replaced by treatment medium. After medium replacement, cells were placed

in the same incubator where they were before, for 24 hours. At the end of that time cells and medium were collected for further analysis. Cell viability and counting was performed with 0.4% trypan blue stain (Sigma-Aldrich, USA) in a LUNA™ Automated Cell Counter (Logos Biosystems, South Korea). The resulting pellets were stored at -80°C.

6. Cell lysis and protein extraction

Cells were lysed using the M-PER solution supplemented with inhibitor cocktail and sodium ortho-vanadate. From the lysate protein quantification was performed using the Pierce™ Microplate BCA Protein Assay Kit – Reducing Agent Compatible (Thermo Scientific, Waltham, MA, USA), which already included standard solutions and the blank. The reading was made at 570nm on Multiskan™ FC Microplate Photometer (Thermo Scientific, Waltham, MA, USA). The amount of protein in the samples was calculated based on the standards, according to the manufacturer's instruction.

7. Lactate dehydrogenase (LDH) enzymatic assay

LDH activity was determined using LDH Enzymatic Assay Kit (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. LDH activity was measured in both extracellular (medium at the end of 24 hours of treatment) and intracellular medium. Levels of LDH activity were measured spectrophotometrically; reading was done at 490nm and at 630 using an Multiskan™ FC Microplate Photometer (Thermo Scientific, Waltham, MA, USA). LDH activity was calculated as units per milligram of protein using the molar extraction factor (ε) and in the end presented as fold variation to control.

8. Cells metabolic activity assay

Metabolic activity of the cells was assessed using tetrazolium dye MTT assay. Living cells are able to reduce tetrazolium dye to its insoluble form formazan, which has a purple color. Briefly, cells were seeded into 96-well culture plate, the same number of cells per well approximately, and maintained until reaching a confluence of 90-95%. Cells were

then treated for 24h with media containing genistein, after that this medium was removed, and cells were washed with PBS. 250µL of ITS medium was added to each well plus 25µL of MTT solution (5mg/mL) and left to incubate in an incubator (37ºC) during 2-4h, until the formation of crystals. The liquid was discarded and 250µl of DMSO was placed to dissolve the formazan crystals and left to agitate for 15 minutes in the dark. 100µL of each well was transferred to a 96-well plate and absorbance was read at 570nm and 655nm in the iMark™ Microplate Absorbance Reader (Bio-Rad, California, USA).

9. Sulforhodamine B (SRB) assay

To evaluate the influence of genistein on the proliferation of hSCs, SRB assay was performed based on (133). Briefly, cells were cultured in a 96-well culture plate, each well with the same number of cells approximately. When cells reached a confluence of 60-70%, they were treated with treatment medium for 24h. At the end of that time cells were fixed with a mixture of 1% acetic acid and 99% methanol, overnight at -20ºC. The next day cells were stained with 0.05% (w/v) SRB dissolved in acetic acid 1% for 1h, the unbound SRB was washed out with acetic acid 1% while the bound SRB was extracted with 10Nm Tris base (pH 10) in a shaker for 10 minutes. A blank was made with only Tris base (pH 10) and absorbance was read at 492nm. To obtain a dose-response, control was taken as 100% and the other groups were evaluated relative to the control.

10. Mitochondrial membrane potential

The membrane potential of the mitochondria in hSCs was measured using the JC-1 fluorescent probe, using a slightly modified method as described in (134). The accumulation of JC-1 within the mitochondria depends on the membrane potential of the same, therefore if mitochondria are functional their membrane potential is high and JC-1 aggregates are formed inside of them. On the other hand, when mitochondria are dysfunctional their membrane potential is low and JC-1 is present mainly in the form of monomers. The ratio between the monomers and the aggregates allows to verify if the mitochondria are functional or not. The greater the ratio between the two, the greater is the depolarization of the mitochondria. hSCs were cultured in a 96-well culture plate with

approximately the same number of cells per well. When cells reached a confluence of about 60-70% they were exposed to treatment medium for 24h, when time was over this medium was taken out and was put 100µl/well of JC-1 staining solution (1µg/mL) dissolved in DMEM:F12 medium (1:1, pH 7.4) containing 1% FBS, during 15 minutes at 37ºC. At the end of that time cells were washed with PBS and given 100µl/well of DMEM:F12 medium (1:1, pH 7.4) supplemented with 1% FBS, the fluorescence intensity was measured immediately at 37°C using Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA). The JC-1 monomers are detected at 485/535nm (excitation/emission), while the aggregates at 550/590nm (excitation/emission).

11. Nuclear magnetic resonance (^{1}H-NMR) spectroscopy and spectral analysis

The spectrum of the extracellular medium was obtained using a Varian 600-MHz spectrometer equipped with a 3-mm indirect detection probe with z-gradient (Varian Instruments, Palo Alto, CA, USA) and quantified according to what is described in (135). Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) for quantification of the following metabolites present in the extracellular medium of cells under study (multiplet, ppm): lactate (doublet, 1.33), alanine (doublet, 1.45), α-glucose (doublet, 5.22), pyruvate (singlet, 2.35), glutamine (triplet, 3.77), acetate (singlet, 1.90). The relative ^{1}H-NMR resonance areas and the concentration of the metabolites present in the medium were calculated as described in (135).

12. Western Blotting

Total proteins from hSCs were isolated with the help of M-PER, and quantified as described above. The resulting protein was mixed with sample buffer (60mM Tris.HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol and 0.01% bromophenol blue (1%)) at a ratio of 1:5, the remaining volume was filled with PBS 1x concentrated to give a final volume of 35µL for each sample. Protein preparations were denatured at 55°C for 10 minutes. Proteins were fractionated in 12.5% polyacrylamide gel for 90 minutes. At the

end of the electrophoresis proteins were transferred to the previously activated polyvinylidene difluoride membrane which was then blocked for 90 minutes at room temperature in a 5% nonfat milk solution. Afterwards, the membrane was incubated overnight at 4°C with rabbit anti-LDH (1:10 000, Abcam). Also, the membrane was incubated with mouse anti-β-actin (1:5000, Thermo Scientific) as protein loading control. After overnight incubation the membrane was incubated with secondary antibody, mouse anti-rabbit IgG-HRP (1:5000, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP (1:5000, Santa Cruz Biotechnology) in case of β-actin. The membrane was read in ChemiDoc™ XRS+ System (Bio-Rad Hemel Hempstead, UK) previously reacted with WesternBright ECL HRP substrate solution (Advansta, California, USA). The density of the bands was measured with the help of Image Lab 5.2 program (Bio-Rad, UK) using the tool that exists in the program for this purpose. Density of the bands obtained was divided by the density of the bands of the corresponding β-actin and expressed as fold variation to control (136).

13. Evaluation of carbonyl groups, lipid peroxidation and nitration

Protein carbonylation, lipid peroxidation and nitration are often used as biomarkers for oxidative stress and they can be evaluated by measuring their resulting products such as 2,4-dinitrophenyl (DNP), 4-hydroxynonenal (4-HNE) and nitro-tyrosine, respectively. Its content in hSCs, after exposure to genistein, was measured by using slot-blot technique with the Hybri-slot manifold system (Biometra, Göttingen, Germany). The resulting PVDF membranes were incubated with the respective antibodies. For the measurement of the carbonyl groups, protein samples were derivate with 2,4-dinitrophenylhydrazine (DNPH) to obtain DNP, according to the method previously described (137). As for lipid peroxidation and nitration analysis, samples were diluted in PBS to obtain a concentration of 12µg/µl. Samples were blotted in the slot-blot and the resulting membranes were incubated overnight at 4°C with respective antibodies, rabbit anti-DNP (1:5000, D9656, Sigma-Aldrich, St. Louis, MO, USA), goat anti-4-HNE (1:5000, AB5605, Merck Millipore, Temecula, USA) and rabbit anti-nitro-tyrosine (1:5000, 9691, Cell Signaling Technology Leiden, Netherlands). Primary antibodies where marked with secondary antibodies anti-goat: rabbit anti-goat IgG-AP (1:5000, A4187, Sigma-Aldrich, USA) and anti-rabbit: goat anti-rabbit IgG-AP (1:5000, sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany), respectively. The reading was done with the help of ECF™ substrate (GE Healthcare,

Buckinghamshire, UK) and the equipment BioRad FX-Pro-plus (Bio-Rad Hemel Hempstead, UK). Densities of the bands were quantified using 1-D Analysis Software from Quantity One (version 4.6.8) (VilberLourmat, Marne-la-Vallée, France).

14. Statistical analysis

The statistical treatment of the data was done using ANOVA, followed by a t-test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± SEM. Differences with $p<0.05$ were considered statistically significant.

IV. Results

1. Genistein is not cytotoxic for hSCs

Due to the similarity of genistein with endogenous estrogens and reported cases in which phytoestrogens led to infertility in animals, we decided to study the effects of genistein on the human male reproductive system. For this work hSCs were chosen since these cells play a very important role in spermatogenesis as they serve as physical and nutritional support for germ cells. In addition, these cells possess ERs, which makes them potential targets of phytoestrogens in the male reproductive system. Firstly, we studied possible cytotoxic effects of genistein on hSCs by choosing some concentrations (in μM: 0; 0.01; 0.1; 1; 10) which has already been mentioned above.

To investigate whether cell death occurred in cells exposed to genistein, we chose to measure LDH activity. This enzyme is responsible for the conversion of pyruvate to lactate and is usually located in the intracellular medium. When cell lysis occurs, this enzyme is released into the extracellular medium, so we measured its activity in the medium to which the cells were exposed for 24h. In the medium, wherewith cells were treated with concentrations of 0.01 μM and 1 μM of genistein, there was slightly lower activity of this enzyme to 0.93 ± 0.06 and 0.97 ± 0.02 fold variation to control, respectively, as compared to the control group which is 1 ± 0.04 (Fig. 4). On the other hand, in the media containing 0.1 μM and 10 μM of genistein there was a slight increase in activity of this enzyme, to 0.97 ± 0.02 and 1.11 ± 0.05, but not significant.

SRB assay, which allows us to detect whether there is an increase or decrease in cell proliferation, has shown, that cells, that were exposed to the medium containing a concentration of 0.01 μM of genistein slightly decreased their proliferation to 91.18 ± 9.14% (fold variation to control) compared to the control group (100 ± 19.87%) (Fig. 4). Interestingly, cells exposed to concentrations of 0.1 μM, 1 μM and 10 μM of genistein increased their proliferation slightly, to 113.96 ± 6.94%, 117.63 ± 4.75%, 108.77 ± 15.96%, respectively (Fig. 4).

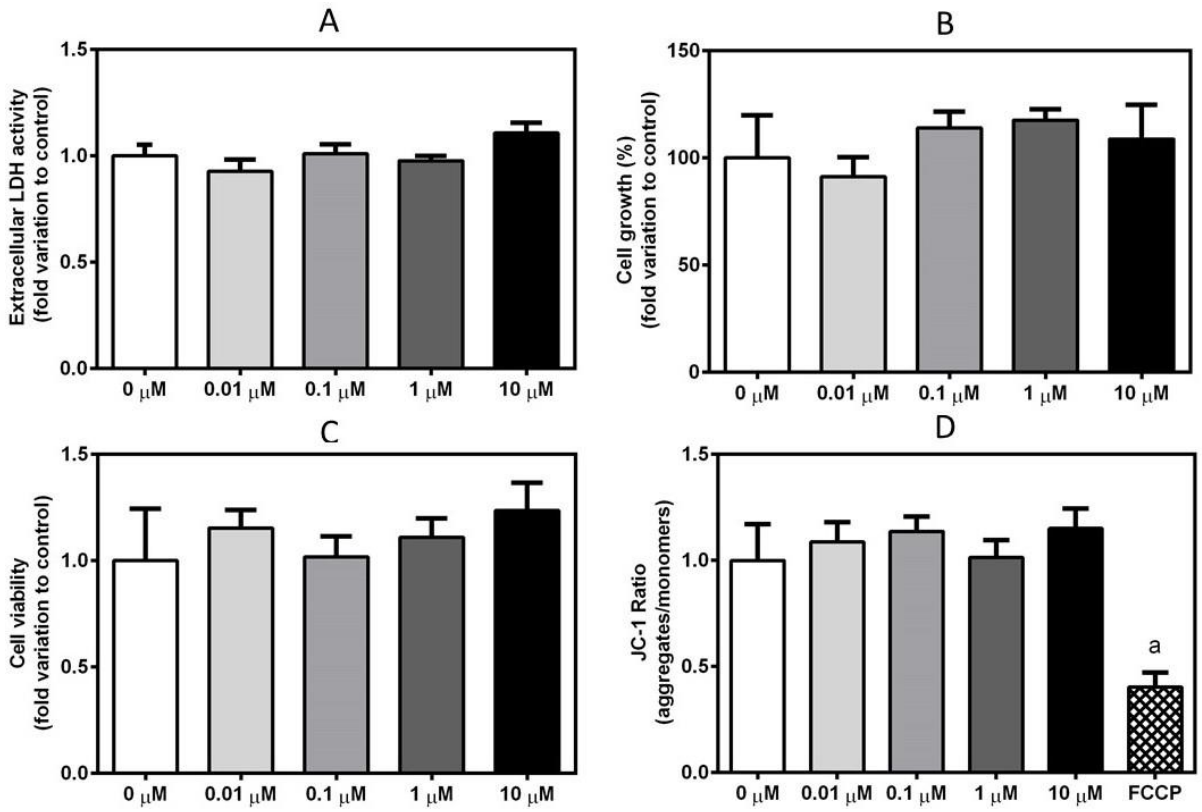


Figure 4 – Effect of different concentrations (0 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM) of genistein on hSCs survival rates (A), proliferation (B), metabolic activity (C) and mitochondrial viability (D). Each of this experiment was conducted separately and values are expressed as fold variation to control. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P<0.05$) are indicated as (a) relative to every group. In this case, graphic D, (a) represents the positive control, these cells were treated with FCCP whose role is to disrupt mitochondrial membrane potential.

In order to determine if cells were metabolically viable, the MTT assay were performed. This test depends on NAD(P)H, which cells can produce only if they are viable, for the reduction of tetrazolium dye to its insoluble form which can be detected due to its purple color. Interestingly, all genistein-treated groups slightly increased their metabolic function to 1.15 ± 0.08, 1.02 ± 0.09, 1.11 ± 0.08 and 1.23 ± 0.12 fold variation to control, after exposure to 0.01, 0.1, 1 and 10 μM of genistein, respectively, when compared to the control group which presented a value of 1 ± 0.24 (Fig. 4).

Another important component in cellular functioning is mitochondria, which are responsible for the production of energy in cells. ATP production is supported by the electron transport chain which is located on the inner membrane of the mitochondria and generates electrochemical proton gradient. This gradient is measured as membrane potential and can be detected with the aid of JC-1 dye. In genistein-treated cells, a slight increase in membrane potential was observed (1.09 ± 0.09, 1.14 ± 0.07, 1.01 ± 0.08, 1.15 ± 0.09 after exposure to 0.01, 0.1, 1 and 10 μM) when compared to the control group (1 ± 0.17), all as fold variation to control, but was not significant (Fig. 4).

All of the alterations mentioned above were not significant, based on the statistical tests performed.

2. Exposure of hSCs to genistein did not alter their lactate production metabolic pathway

Since genistein is not toxic to hSCs, we studied if it affected the metabolism of these cells and, so we choose one of the main metabolic pathway for spermatogenesis, glycolysis, which can be summarized as production of lactate from glucose.

Lactate is crucial since it is exported to the extracellular medium, where it is uptaken by germ cells and used as source of energy. Conversion of lactate is mediated by the enzyme LDH and therefore we measured its intracellular activity. Cells exposed to 0.01 μM concentration slightly decreased the amount of LDH activity (0.72 ± 0.35, fold variation to control) when compared to the control group (1 ± 0.50) (Fig. 5). In cells exposed to genistein in the concentrations of 1 and 10 μM the decrease in LDH activity was most noticeable, 0.13 ± 0.04 and 0.09 ± 0.05, respectively (Fig. 5), however not significant. Nevertheless, we cannot completely ignore that, with the higher concentrations of genistein, LDH activity tended to decrease. Also, we measured LDH protein expression, which increased slightly in the groups exposed to 1 μM and 10 μM, 1.29 ± 0.31 and 1.37 ± 0.29 as fold variation to control, respectively. Group treated with 0.01 μM (0.93 ± 0.19, fold variation to control) of genistein remained closer to the control group (1 ± 0.22), no significant variations were found.

An analysis of ^{1}H-NMR allowed us to analyze the amount of metabolites present in the medium. We selected the most relevant for these cells taking in consideration previous studies from our team. Glucose uptake, decreased from 14.78 ± 7.17 mmol/10^{6} cells in the control group, to 4.48 ± 0.83 mmol/10^{6} cells in cells exposed to 0.01 μM of genistein and to 6.40 ± 1.54 mmol/10^{6} cells in 10 μM group (Fig. 6). Interestingly, the group treated with 1 μM of genistein increased its uptake to 21.45 ± 10.97 mmol/10^{6} cells (Fig. 6). The glucose in turn is converted to pyruvate, uptake of which did not vary significantly between the groups, only in the group treated with 1 μM of genistein, was observed a tendency to increase (0.88 ± 0.37 mmol/10^{6} cells) with respect to the other groups of cells exposed to 0, 0.01 and 10 μM (0.60 ± 0.26, 0.61 ± 0.16 and 0.50 ± 0.18 mmol/10^{6} cells, respectively) (Fig. 6). Pyruvate is converted to lactate which is then exported and consumed by developing germ cells. Lactate production slightly increased in groups treated with 1 μM

and 10 µM of genistein, to 15.92 ± 3.61 and 17.37 ± 4.13 mmol/10^6 cells, respectively, compared to the control group that produced 15.61 ± 3.60 mmol/10^6 cells (Fig. 6). Groups treated with 0.01 µM increased its lactate excretion little more than the other groups, to 18.03 ± 3.35 nmol/10^6 cells (Fig. 6). However, pyruvate can also be converted to alanine, whose production also did not have significant variations relative to the control group (0.24 ± 0.11 mmol/10^6 cells), only the group treated with 0.01 µM had a slight increase in the production of pyruvate (0.37 ± 0.09 mmol/10^6 cells) (Fig. 6). Based on statistical tests, differences found are not significant.

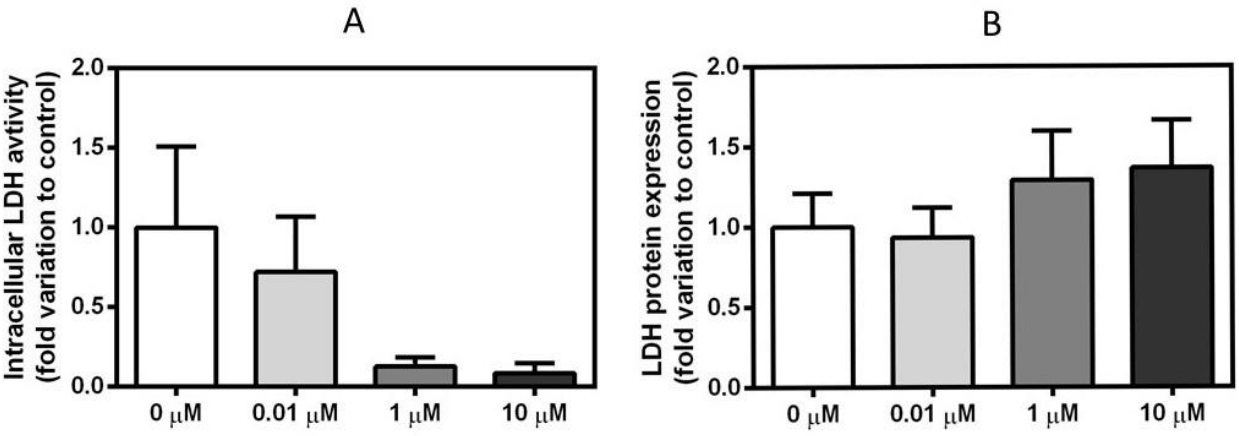


Figure 5 – Variation on lactate dehydrogenase (LDH) intracellular activity (A) and its protein expression (B) in hSCs exposed to different genistein concentrations (0 µM, 0.01 µM, 1 µM, 10 µM). Different experiments results are presented together. Results are presented as fold variation to control and as mean ± SEM (n=5 for each condition).

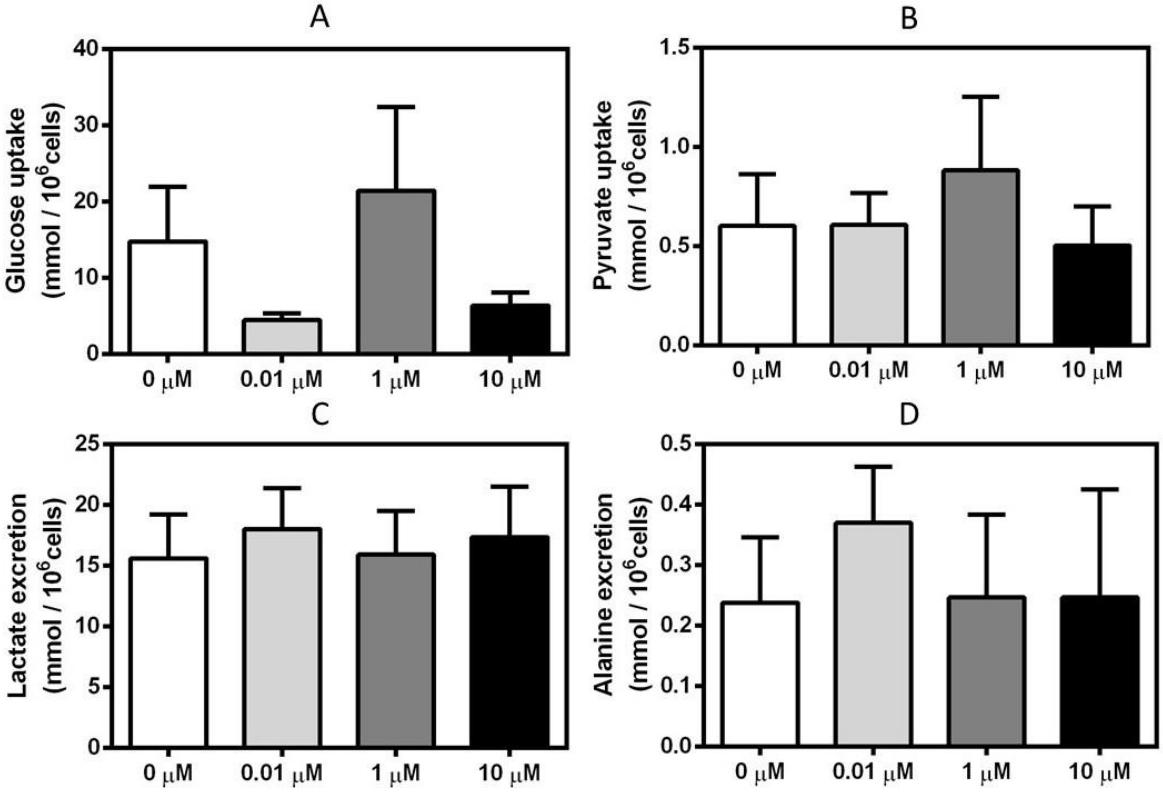


Figure 6 – Variation on glucose uptake (A), pyruvate uptake (B), lactate excretion (C) and alanine excretion (D) in hSCs exposed at different genistein concentrations (0 μM, 0.01 μM, 1 μM, 10 μM). Consumption and excretion are presented in mmol/10^6 cells. Results are expressed as mean ± SEM (n=6 for each condition).

3. Genistein did not alter glutamine and acetate uptake by hSCs

Other components that can be found in the extracellular medium and that are crucial for hSCs metabolism are glutamine and acetate. These molecules participate in different metabolic pathways and are also used for energy production by entering the tricarboxylic acid (TCA) cycle via Acetyl CoA. In genistein-treated hSCs acetate uptake had a decrease which was more evident in groups treated with 0.01 and 10 μM (0.03 ± 0.04 and 0.03 ± 0.02 mmol/10^6 cells, respectively). On the other hand, the group treated with 1 μM (0.08 ± 0.14 mmol/10^6 cells) was very close to the control group (0.10 ± 0.08 mmol/10^6 cells) (Fig. 7). In the case of glutamine, a decrease in uptake was observed in groups treated with 0.01 and 10 μM of genistein, to values 0.65 ± 0.56 and -0.05 ± 0.29 mmol/10^6 cells, respectively, while the group treated with 1 μM increased its uptake to 2.44 ± 1.58 mmol/10^6 cells, compared to the control group that had a value of 1.53 ± 1.04

mmol/10^6 cells (Fig. 7). Nevertheless, the differences found were not statistically significant.

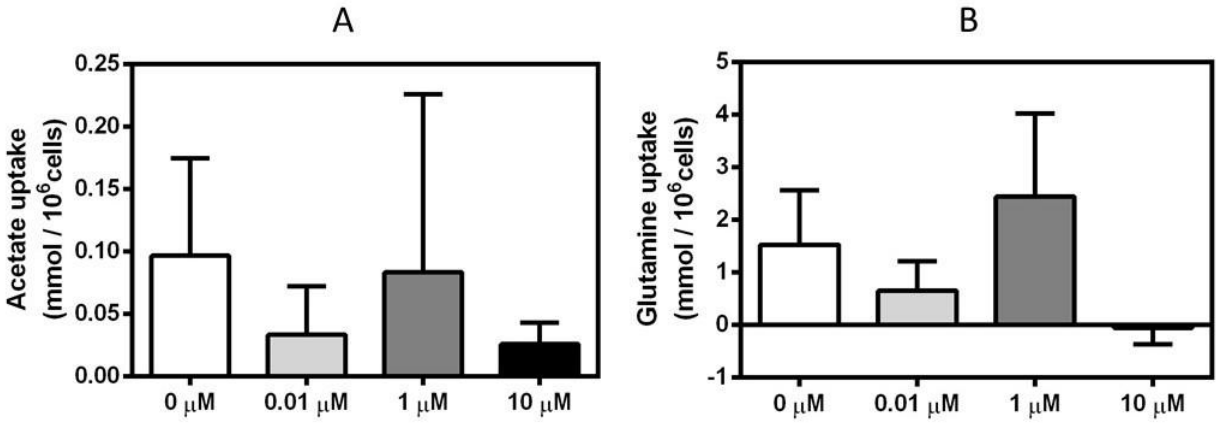


Figure 7 – Variation on acetate (A) and glutamine (B) uptake by hSCs exposed to different genistein concentrations (0 μM, 0.01 μM, 1 μM, 10 μM). Uptake is presented in mmol/10^6 cells and results are expressed as mean ± SEM (n=6 for each condition).

4. Exposure of hSCs to genistein did not alter protein carbonylation and nitration but slightly increased lipid peroxidation

Cellular metabolism gives rise to reactive oxygen species (ROS) that can cause cell damage at different levels if they are not efficiently eliminated by the cell, this is called oxidative stress (OS). Lipid peroxidation, carbonylation and nitration of proteins are strong biomarkers of OS hence they are often used to evaluate it. Products of these reactions can be detected and quantified thus allowing us to evaluate the severity of OS. In order to quantify the protein carbonylation, DNP groups are detected. They are a result of the derivatization mentioned above, and in the genistein-treated hSCs there was no significant change in their amount relative to the control group (1 ± 0.10), only a slight decrease (Fig. 8). Groups treated with 0.01, 1 and 10 μM had values of 0.85 ± 0.02, 0.88 ± 0.08 and 0.84 ± 0.07, fold variation to control, respectively (Fig. 8). Another effect of OS on proteins is their nitration, in this case it is evaluated the amount of nitrotyrosine, which, as happened with carbonylation, did not alter much, only slightly decreased to 0.94 ± 0.07 and 0.90 ± 0.08 fold variation to control, in groups of hSCs treated with 1 μM and 10 μM respectively, compared to the control having a value of 1.04 ± 0.07 (Fig. 8). Only the group treated with 0.01 μM had a slight rise (1.07 ± 0.06, variation to control) but did not

differ that much from the control group (Fig. 8). As for lipid peroxidation, slight variations were detected. There was a slight increase, in cells treated with genistein, to 1.17 ± 0.21, 1.22 ± 0.20 and 1.29 ± 0.27 fold variation to control, in groups treated with 0.01 μM, 1 μM e 10 μM, respectively (Fig. 8), compared to the control group whose value was 1 ± 0.13. However, statistical tests considered these variations as not significant.

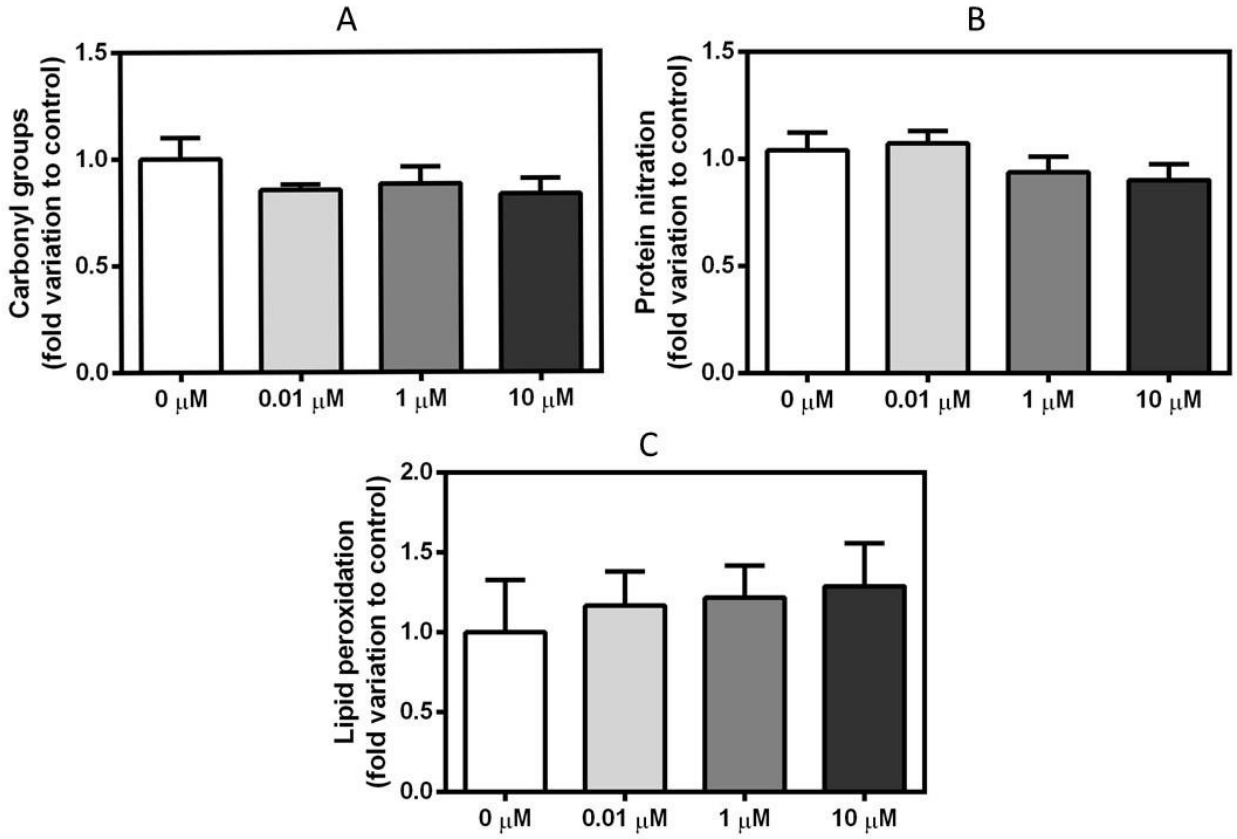


Figure 8 – Effect of genistein, at different concentrations (0 μM, 0.01 μM, 1 μM, 10 μM), on oxidative stress in hSCs. Here is represented data from independent experiments, which sums protein level of carbonylation, nitration and lipid peroxidation. Results are expressed as mean ± SEM (n=5 for each condition) and as fold variation to control.

V. Discussion

Phytoestrogens are compounds produced by plants for their defense against pathogens (138), but their chemical similarity with animal estrogens aroused the interest of scientists for their possible effects in health. Many plants have these compounds, however, of those that are consumed by humans stands out the bean family which present a higher percentage of phytoestrogens (47, 139). Within this family, soy has become the focus of intense research due to large amounts of phytoestrogens that it possesses relative to other beans, it is especially rich in genistein and daidzein (74, 140). However, daidzein has less affinity with ERs that genistein (47, 57), which made it the main phytoestrogen of interest to scientific studies that aim to study hormonal-dependent processes.

The similarity of genistein to animal estrogens has not immediately aroused much interest. This changed when it has been reported a case where phytoestrogens led to infertility in sheep grazing in fields that had predominantly clover (6), which are rich in different types of phytoestrogens, including genistein although it is not the predominant one here. This study, consubstantiated by many others showing both, positive and negative effects in animal health, Table 1 and (141, 142), led to the discussion concerning the consumption of some products of plant origin present in the human diet, which are very rich in phytoestrogens. The safety consumption of those products has been a matter of intense debate. Thus, several studies have been performed in an attempt to understand its influence on health. For instance, several studies already demonstrated some mechanisms of action of phytoestrogens in systems and organs, including the discovery that phytoestrogens are able to inhibit tyrosine kinase and topoisomerase II (81-85), which are important proteins in cell replication and, therefore, potential targets in cancer treatment. Meantime other beneficial functions have been attributed to phytoestrogens due to their estrogenic action, although a weak one. It has been shown that it is capable of producing beneficial effects in estrogen-regulated systems, such as bone remodeling, thereby preventing osteoporosis, alleviating menopausal symptoms, reducing the risk of cardiovascular and cancer diseases, delaying skin aging, boosting the immune system, and even improving memory. However, its action on the reproductive system remains unclear even after the reported cases of infertility in sheep discussed earlier. Therefore, it is crucial to unravel the effects of genistein in the reproduction, particularly in male

reproduction taking in consideration the current research that highlights a growing influence of estrogens for the establishment of the male reproductive potential.

The overwhelming majority of studies were performed on rats/mice, in which they were subjected to different doses and types of phytoestrogens, at different stages of development. However, after summarizing these studies it is difficult to reach to a conclusion concerning the safety of phytoestrogens, and genistein, in particular, and their influence on human and animal fertility. The results attained in much of those studies are contradictory; one part of the studies claims that there are not any significant alterations on the reproductive system of the individuals studied while others concluded that there are some disturbances in the reproductive potential of the individuals studied, Table 1. Nevertheless, we must highlight that even evolutionarily close organisms have differences and this can lead to different reactions to the same compound. This was well shown in knockout animals for ERs and aromatase. Animals and humans sharing the same deficiency of either ER or aromatase enzyme had different phenotypes, although with some similarities, as mentioned earlier. In turn, human studies are scarce and mainly based on surveys and analysis of data obtained from infertility clinics, which may not be very reliable since other external and even internal factors may be the cause of abnormalities in the reproductive system.

To study the influence of genistein on male fertility human Sertoli cells as model (143) were chosen. These cells serve as physical and nutritional support for spermatogenesis, to germ cells, providing them nutrients and metabolic intermediates besides forming blood-testis barrier that protects them and creates an environment favorable to their development (144-147). Another important factor in the choice of these cells is the fact that they possess ERs and our group has previously described that their metabolism is influenced by estrogens (148-150), which makes them potential targets of phytoestrogen.

Our first approach consisted in studying if genistein was cytotoxic to hSCs. Our results showed that this phytoestrogen is not toxic to the hSCs as measured by the activity of the LDH in the extracellular medium. The normal location of this enzyme is in the intracellular environment, so if the amount of it increases in the extracellular medium this would be a sign of cell death. However, our results show that *in vitro* exposure of hSCs to the selected concentrations of genistein does not increase cell death. Differences were also not found in the proliferation of genistein-exposed hSCs nor in the general metabolic activity as measured by the SRB and MTT test, respectively. This is in accordance with several studies that suggest the safety of genistein *in vitro* or even report

a cytoprotective effect. This has been associated with the antioxidant properties of this phytoestrogen (87, 88, 117, 118).

Another important organelle for cellular metabolism is the mitochondria, since they produce most of the cellular energy. Thus, although no differences were detected concerning the overall metabolic activity in genistein-exposed cells, we measured the mitochondrial membrane potential to access the number of viable mitochondria. Interestingly, our results suggest that the doses used in this study are not able to modulate mitochondrial membrane potential. This may be due to several factors, including the specificity of these cells. The SCs mainly produce lactate and acetate from glucose and only rely on the mitochondrial activity to support their own energetic needs (151) thus, since there is no alteration on cells proliferation nor in cell death, it would be expectable that mitochondria would remain functional.

One of the main functions of hSCs is the nutritional support of spermatogenesis. This is mainly done by glycolysis and further conversion of pyruvate to lactate and other metabolites needed to the development of germ cells. Although no differences were detected in metabolic activity and mitochondrial membrane potential, we studied some mechanisms associated with glycolysis and lactate production. In order to evaluate the amount of metabolites of this pathway we used ^{1}H-NMR analysis of the extracellular media. No significant differences were detected in the analyzed metabolites. Nevertheless, although glucose uptake did not show any significant variation, we detected a tendency to decrease after exposure to genistein except at the concentration of 1μM in which the uptake was slightly higher than in the group without genistein. The glucose taken by the cells is then converted to pyruvate, but pyruvate can also be taken from extracellular sources, including the media. Our results showed that genistein, in the doses tested, did not alter the uptake of pyruvate from the extracellular media though the cells treated with 1μM presented a slight tendency to increase in its uptake. Pyruvate can either be converted to lactate or into alanine or proceed to the mitochondria where it will enter the TCA cycle for further energy production. For germ cells, lactate production is crucial since they use it as an energy source. In fact, developing germ cells cannot metabolize glucose on their own and rely on the lactate produced by SCs that is exported to the lumen of the seminiferous tubules where these cells are located. Lactate excretion remained equal between groups as well as alanine excretion though a slight tendency in alanine excretion increase was observed in the group treated with 0.01μM. This is concomitant with the lack of effect in glucose and pyruvate uptake. We further studied if genistein could interfere with intracellular LDH activity or expression. LDH can convert

pyruvate to lactate in a bidirectional way. Our results show that genistein, in the tested concentrations, did not alter the activity nor the expression of this enzyme although a tendency to decrease the enzyme activity was observed as the genistein concentration increased. Curiously the inverse was observed with the expression of this protein. Nevertheless, these results are non-significant which suggests that further experiments may be needed to consolidate this tendency as irrelevant or a crucial point for the action of genistein in the male reproductive tract. Taken together, our results suggest that lactate production and glycolysis, both essential processes for spermatogenesis, were not influenced by the presence of genistein. Other important metabolites for cellular metabolism are glutamine and acetate since they enter lipid and protein metabolism and can also proceed to TCA cycle through Acetyl-CoA, which results in an increase in energy production. Our results suggest that the exposure to genistein in the concentrations tested did not change the uptake of these compounds. Again, it was detected a trend to a decreased uptake in genistein-treated groups except for the cells treated with 1μM, where glutamine slightly increased its uptake. This suggests that further studies may be needed to completely unveil the effect of genistein in these cells as an increase in the number of primary cells attained from more biopsies may increase the statistical power of the results and consolidate the findings.

Cellular metabolism also generates reactive oxygen species that, if not effectively eliminated by the cell, will cause irreversible damages in the same and can even lead to its death. It has already been reported that phytoestrogens have antioxidant properties (87, 88, 117, 118). Thus, we evaluated the effect of genistein in the oxidative profile of hSCs. For this we evaluated protein carbonylation and nitration, and also lipid peroxidation. Our results showed that genistein in the tested concentrations did not changed the oxidative profile of hSCs. This is concomitant with all the previous results showing that genistein had no effect on the metabolic activity, proliferation, mitochondrial activity nor in the glycolytic activity of hSCs.

Overall, our data clearly shows that the *in vitro* exposure of hSCs to genistein in the selected concentrations is not cytotoxic and does not changes the proliferation or metabolic activity of these cells, including their major function in the nutrition support of spermatogenesis. Though all the small changes were non-significant, it is important to note that further studies will be necessary to characterize the mechanisms of action of genistein in hSCs and how it can change the metabolic functioning of these cells. Therefore, at this point, our results point towards the conclusion that genistein can be

considered safe but taken in consideration the fact that this is the first work focused on the effects of genistein in hSCs, cautions should be taken.

VI. Conclusions and Future Perspectives

Interest in phytoestrogens has grown exponentially in the last decades due to their beneficial properties on human health. Nonetheless, their similarity to animal estrogens, and the discovery of cases where exposure to phytoestrogens was associated with disturbed fertility in animals, has given rise to concerns about their safety and potential undesirable effects on reproductive system, which is highly regulated by estrogens even in men.

Information from the current literature is contradictory and therefore it is difficult to reach to a conclusion. This difference between studies is due to several factors and studies on animals cannot be directly extrapolated to humans due to the biological differences between the organisms. For example, in animals such as boars and roosters isoflavones appear to have beneficial effects on fertility and sperm parameters (141, 142) while in sheep it was associated with infertility, as mentioned above. As previously mentioned, animal studies can only serve as a basis to study the molecular mechanisms and mechanisms of action of phytoestrogens in mammals. Since human studies are scarce and mostly based on surveys and clinical data analysis, we studied (*in vitro*) the effects of genistein, one of the most consumed phytoestrogen by humans, on human Sertoli cells, which are responsible for the physical and nutritional support of spermatogenesis.

Taken together, our results show that genistein does not induce any significant effects on hSCs viability, metabolism and oxidative stress, however this does not clearly suggests that phytoestrogens, and genistein itself, are safe for the reproductive potential of the individuals. Further studies will be needed, particularly to consolidate the findings of the differences that were not considered significant by the statistical analysis. Those tendencies, that could influence the overall conclusions, need and deserve further studies .

Also, surveillance of SIF-fed children is necessary since it is at this point in life (neonatal) that the testes are structurally organized, and spermatogonia and Sertoli cell numbers are established, which will play a significant role on spermatogenesis when it will begin at puberty.

In conclusion, it is therefore necessary a greater investment on study the action of genistein, and other phytoestrogens, on human health, particularly on male fertility and the

possible effects on the quality of reproduction, because they are indeed compounds with great therapeutic potential and with possible beneficial effects on the body.

VII. References

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